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Carbohydrates of the Organic Shell Matrix and the Shell-Forming Tissue of the Snail *Biomphalaria glabrata* (Say)

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Abstract. Sulfated carbohydrates may play a role in the biomineralization of the molluscan shell. The carbohydrates of the extracted water-insoluble organic shell matrix (IM) of the freshwater snail *Biomphalaria glabrata* were identified as glucose, mannose, galactose, and *N*-acetyl-glucosamine, whereas the water-soluble organic matrix (SM) additionally contained *N*-acetyl-galactosamine. A specific lectin binding pattern of the matrix was obtained. One prominent protein of the SM, with a size of 19.6 kDa and a pI of 7.4, was shown to be a glycoprotein with terminal glucosyl or mannosyl moieties. The acidic constitutents of the matrix showed a variety of possible terminal sugars, indicating a heterogenous mixture of proteoglycans or glycosaminoglycans (GAGs) and glycoproteins.

At the shell-forming mantle edge, an alcian-blue-positive material was observed in the periostracum groove (PG), the belt, and apically in the cells of the outer mantle epithelium (OME). With the help of lectins, all sugars in question were detected in the PG and the belt, whereas the OME was bound by glucose/mannose- and GlcNac-specific lectins only. Although the complete set of GAGs will be produced in the PG and the belt, a very acidic fraction of GAGs and the 19.6-kDa protein can also be delivered by the OME.

Introduction

The biomineralization of the molluscan shell is controlled to a high degree by the organic shell matrix. The processes that have to be controlled are the crystal nucleation and then the modification, morphology, and size of the growing crystals. Macromolecules from the molluscan water-soluble organic matrix (SM) can modify calcium carbonate crystals *in vitro* (Belcher *et al.*, 1996; Falini *et al.*, 1996). Furthermore, acidic macromolecules from the SM can influence the crystal morphology by stereoselective binding (Addadi and Weiner, 1985).

The shell-building tissue of molluscs, the mantle, secretes all shell material into the extrapallial space below the periostracum. Here, the shell is formed in a way that is still scarcely understood. The mantle edge of pulmonates can be morphologically and physiologically divided into five zones (Timmermanns, 1969; Bielefeld *et al.*, 1993a), which should reflect functional units. If the exact function of each unit could be defined successfully, the sequence of the functional units would allow us to draw conclusions about the chronology of the mineralization process. Therefore, it is of special interest to localize the production site of specific matrix components at the mantle edge.

In the past, analysis of molluscan shell matrices concentrated mainly on the proteins, and minor attention was paid to its carbohydrates. The interest focused primarily on chitin, which is found in the water-insoluble organic matrix (IM) of many molluscs (*e.g.*, Poulicek *et al.*, 1991) and could play a special role in the structural framework of the shell (Weiner and Traub, 1984; Falini *et al.*, 1996). But other carbohydrates, especially in the SM, should be investigated because they might provide sulfate groups, which possibly concentrate calcium in the shell (Addadi *et al.*, 1987).

In the SM of several oysters, hexosamines or hexoses and uronic acids are found in combination with sulfate (Crenshaw, 1972; Samata and Krampitz, 1982). Similarly,

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Abbreviations: GAG, glycosaminoglycan; IM, water-insoluble organic matrix; IME, inner mantle epithelium; OME, outer mantle epithelium; PG, periostracum groove; SM, water-soluble organic matrix.

in the SM of the aragonitic cross-lamellar structured shell of the freshwater gastropod *Biomphalaria glabrata,* the amounts of hexosamines, hexoses, uronic acids, and sulfate were 8.8%, 12.7%, 2.1%, and 14% (w/w), respectively (Marxen and Becker, 1997). After SDS-electrophoresis, the SM showed considerable material stained positively with Stains-all and alcian blue. These stained areas probably represent glycosaminoglycans (GAGs) bound to proteins. Prominent among the matrix proteins of *B. glabrata* was one that, with a size of 19.6 kDa, an isoelectrical point of 7.4, and a hydrophobic *N*-terminus (Marxen and Becker, 1997), might be a glycoprotein.

Our approach in this study was the biochemical identification of the carbohydrates in the organic shell matrix of *B. glabrata,* and the detection of glycoproteins. We expected that the histochemical localization of carbohydrates at the shell-building mantle edge would reveal where the glycosylated components of the organic shell matrix are secreted.

Materials and Methods

Animals

Between 200 and 300 snails of the species *Biomphalaria glabrata* (Say, 1818) (Basommatophora, Planorbidae) were kept in 80-l aquaria with a water exchange of about 200 l of dechlorinated tap water per day. The water was preheated to 28 ± 1°C, and the illumination cycle was 12 h light to 12 h dark. The animals were fed *ad libidum* with a food prepared according to Standen (1951).

Extraction of the shell matrix

The organic matrix of the shell was extracted as described elsewhere (Marxen and Becker, 1997). Briefly: 100 g of powdered shell, including the periostracum, was suspended in 50 ml 10^{-5} *M* HCl. The shell powder was decalcified with HCl under continuous stirring at +4°C, the pH never dropping below 5.0. Each time the volume reached 250 ml, the pH was adjusted to 7.4 and the preparation was allowed to rest for 30 min., then centrifuged for 20 min. at 16,000 × *g*. The supernatants were stored, and the preservatives NaN_3 and AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride) added. The pellet remaining after decalcification was washed, lyophilized, and termed insoluble matrix (IM). All supernatants from the decalcification were combined and dialyzed with 20 changes of the sixfold volume against bidistilled water. Material that precipitated during dialysis was removed by centrifugation at $10^5 \times g$ for 30 min. This pellet had an intermediate degree of solubility in water and was not further investigated. The volume of the supernatant of the 10^5-*g* centrifugation was reduced by lyophilization to 20 ml and further desalted on a P-2 column (Bio-Rad). The void volume was lyophilized and termed the soluble matrix (SM).

Determination of alkali-resistant hexosamines

Protein was removed by boiling the matrix samples for 6 h in 0.5 *M* NaOH and then washing with 1 *M* HCl, 95% (v/v) ethanol, acetone, and 100% ethanol (Jeuniaux, 1963). In the alkali-resistant residue, hexosamines were quantified using Ehrlich's reagent as described by Elson and Morgan (1933) and modified by Kabat and Meyer (1961).

Infrared spectrometry

A KBr pill of the soluble or insoluble organic matrix of *B. glabrata* was prepared according to the method of Günzler and Böck (1983), using 1% of dried organic shell matrix, and analyzed on a Perkin Elmer 841 infrared spectrometer. For a better detection of chitin, protein was removed from some samples of the IM, as described above.

Gas chromatography

Sugars were identified with gas chromatography (Hewlett Packard, Model 437A) using a 10 m × $^1/_8$ inch column packed with 3% silicone OV 225 on Chromosorb W HP 80–100 mesh. Samples were prepared according to Chaplin (1982). An optimal methanolysis was achieved with 2 *M* HCl for 16 h at 85°C. The dry, methanolyzed samples were dissolved in 500 μl waterfree methanol and mixed with 10 μl pyridine and 50 μl acetic anhydride. After 15 min of incubation at room temperature, the samples were dried overnight (Kozulic *et al.,* 1979). The reacetylated samples were mixed with 100 μl silylation reagent (trimethylsilyl imidazole : *N,O*-bis-(trimethylsilyl)-acetamide : trimethylchlorosilane = 3:3:2) (Sweeley *et al.,* 1963), and incubated for 15 min at 70°C and for 45 min at room temperature. Inositol was used as an internal standard. Injector and detector temperatures were 250°C, and the elution program was 2 min at 120°C, rising by 6°C/min. to 220°C.

Lectin binding to the soluble matrix

For the visualization of the lectin binding to the SM, an assay analogous to an ELISA was used. Between all subsequent steps the microtiter plate was washed three times with wash solution (150 m*M* NaCl + 0.1% (v/v) Tween 20), 200 μl/well. A 96-well microtiter plate was coated with SM, each well with 10 μg protein/50 μl 0.2 *M* sodium carbonate buffer, pH 9.5. Unspecific binding was blocked with 200 μl/well blocking buffer: 1% (w/v) carbohydrate-free BSA in 50 m*M* Tris/HCl + 150 m*M* NaCl, pH 7.5. Stock solutions of the biotinylated lectins (Vector, Burlingame, CA) were prepared in a concentration of 1 mg/ml in 10 m*M* PBS + 150 m*M* NaCl, pH 7.4 + 0.25% (w/v) BSA + 0.1% (w/v) NaN_3. The lectins of the stock solutions were diluted 1:100 with dilution buffer:

50 m*M* Tris/HCl + 150 m*M* NaCl, pH 7.5 + 0.5% (w/v) BSA + 0.05% (v/v) Tween 20. This lectin test solution (50 μl/well) was incubated with the SM for 30 min at 37°C. Thirty minutes prior to use, the complex of avidin and biotinylated peroxidase (ABC) (Vector) was prepared; each component was diluted 1:200 in the dilution buffer. The ABC was incubated 50 μl/well for 30 min at 37°C. As a final step, 50 μl/well of the substrate solution were incubated for 30 min at 37°C in the dark. ABTS (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) in a concentration of 95.3 mg/100 ml substrate buffer was used as a chromogene. The substrate buffer was 50 mg sodium perborate trihydrate + 836 mg citric acid monohydrate + 1068 mg disodium hydrogen phosphate dihydrate filled up to 100 ml with bidistilled water, pH 4.5. The optical densities at 405 nm were measured with a microplate reader. The quality of all lectins was tested with appropriate neoglycoproteins, which replaced the SM in these controls. The unspecific binding of the lectins to the microtiter plate was tested, leaving some wells uncoated. The specificity of the lectin binding to the SM was tested by preincubating the lectins with suitable carbohydrates for 30 min. The binding was considered specific only when an inhibiting effect was observed.

Lectin binding to the insoluble matrix

The investigation of the lectin binding to the IM was carried out in small plastic centrifuge vials. All buffers and reagents were as described for the lectin binding to the soluble matrix, except that the lectin stock solutions were diluted 1:200, and the ABC-complex 1:400, with dilution buffer. The sample solution was 1 mg/ml dilution buffer. The vials were filled completely with blocking buffer, kept overnight at 4°C, and then emptied. Next, 25 μl of the constantly stirred sample solution plus 100 μl of diluted lectin was placed in the emptied vials and incubated for 30 min at 37°C. The vials were washed three times with 300 μl wash solution and centrifuged at 8000 $\times$ *g* for 3 min in between. A 100-μl sample of the ABC was incubated and washed in the same way. After incubation with 100 μl of substrate solution for 15 min, 50 μl was pipetted into a microplate for measuring the optical densities at 405 nm.

Isoelectrical focusing

The isoelectrical focusing (IEF) was performed according to the recommendations of Serva (Heidelberg, FRG). Servalyt precotes (125 $\times$ 125 mm) with a polyacrylamide layer of 150 μm and a pH gradient from pH 3.0 to 10.0 were used. After 30 min of prefocusing, the samples were loaded and separated for 1.5 h with a maximum of 2000 V, 6 mA, and 4 W at 4°C.

Lectin binding to the isoelectric focusing gel

When the IEF run was finished, biotinylated lectins were applied directly to the gel according to a procedure modified from Allen *et al.* (1976). The gel was fixed with 12.5% (v/v) TCA for 15 min and washed three times with wash solution (see *Lectin binding to the soluble matrix*). The gel was incubated under constant shaking for 1.5 h at room temperature with lectins from the stock solutions, diluted 1:100 with 50 m*M* Tris/HCl + 150 m*M* NaCl, pH 7.5, + 0.1% (v/v) Tween 20, and washed three times. ABC, diluted 1:50 in the same solution 30 min prior to use, was incubated for 1 h. AEC (3-amino-9-ethylcarbazole), dissolved in DMF (dimethylformamide) 4 mg/ml, was used as a chromogene. The freshly prepared substrate solution contained 76 ml of 0.05 *M* acetate buffer, pH 5.0, 4 ml of AEC in DMF, and 400 μl 3% (v/v) H_2O_2. The gel was incubated in the dark with the substrate solution until intense red bands appeared.

Histological detection of mucus

Pieces of the mantle edge were fixed by three methods. Method 1: Fixation for 28 h at room temperature in 2% (v/v) glutardialdehyde in 0.05 *M* cacodylate buffer, pH 7.4. Method 2: Fixation for 24 h at room temperature in 4% (v/v) formaldehyde in 0.067 *M* phosphate buffer, pH 7.4, with 0.5% (w/v) cetylpyridinium chloride added. Method 3: Fixation for 18 h at 4°C in 4% (v/v) formaldehyde in picric-acid-saturated ethanol with 5% (v/v) acetic acid. After embedding in paraplast, sections of 5-μm thickness were cut, and the Paraplast was removed. Mucus and mucus cells were stained with 1% (w/v) alcian blue 8GX in 3% (v/v) acetic acid at pH 2.5 and counterstained with either Kernechtrot or PAS. For the differentiation between carboxylic and sulfate groups, the alcian blue staining at pH 1.0 (Lev and Spicer, 1964) and the critical electrolyte concentration (Scott and Dorling, 1965) were carried out.

Lectin histochemistry

Pieces of the mantle edge were fixed for 26 h at room temperature in 2% (v/v) formaldehyde in a solution of 25% (v/v) ethanol, 25% (v/v) ethyl acetate, 5% (v/v) acetic acid, and 0.5% (w/v) picric acid. From sections of 7-μm thickness, embedded in Paraplast, the Paraplast was removed, and the endogenous peroxidase was blocked with 1% (v/v) H_2O_2 in 100% methanol. Unspecific binding was blocked with 2% (w/v) BSA in PBS (150 m*M* NaCl, buffered with 10 m*M* phosphate, pH 7.4). The sections were incubated with biotinylated lectins (Vector Laboratories, Burlingame, CA), in dilutions from 1:50 to 1:1600 in PBS, pH 7.4, containing 0.25% (w/v) BSA and 0.1% (w/v) NaN_3 for 18 h at 4°C in a moistened chamber. After careful rinsing with PBS, the avidin-biotin-peroxi-

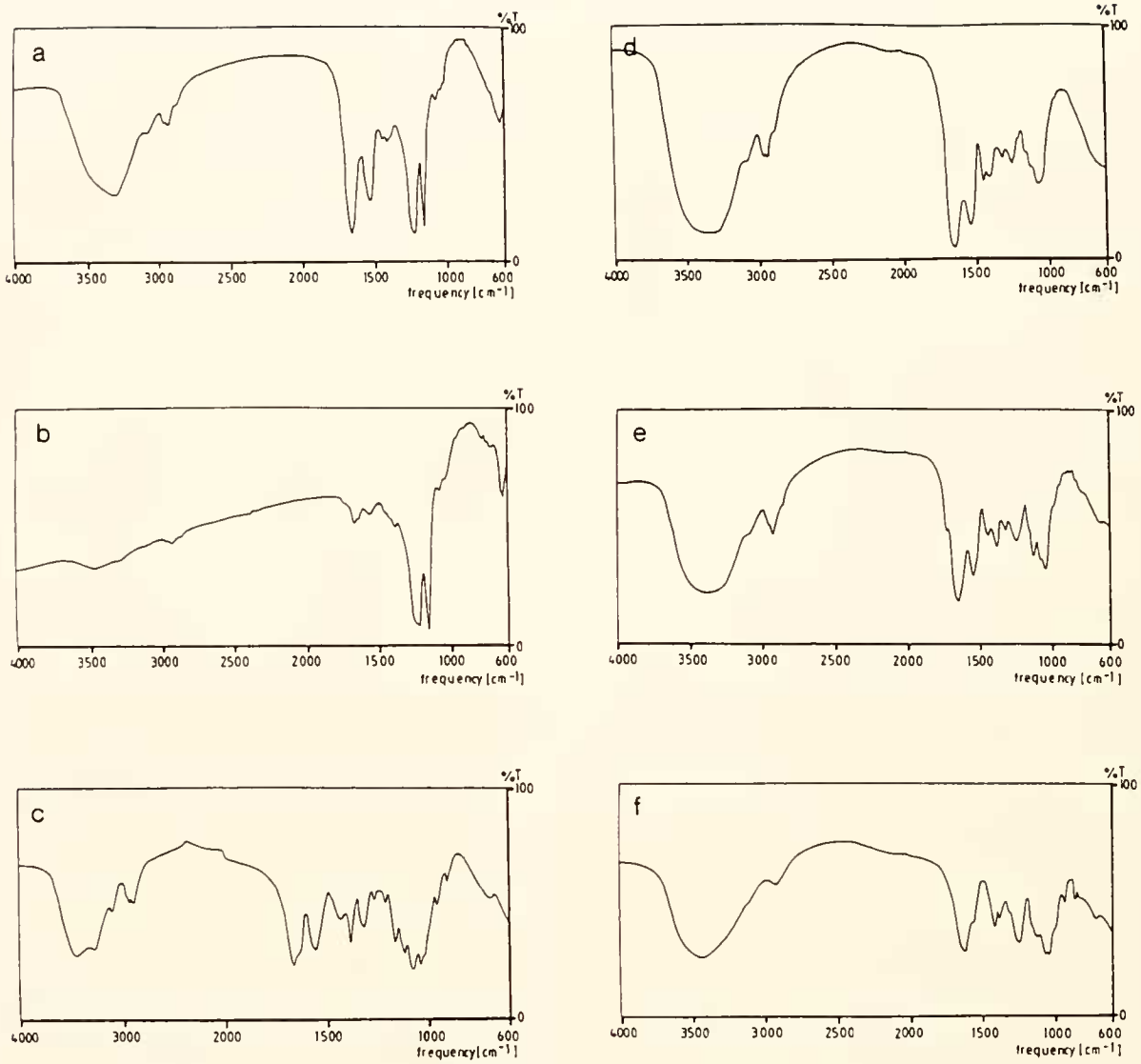

Figure 1. The infrared absorption spectra from (a) the extracted water-insoluble organic matrix fraction of the *Biomphalaria glabrata* shell; (b) the alkali-treated, protein-free water-insoluble organic matrix; (c) chitin from crabshell; (d) the extracted water-soluble organic matrix fraction of the *B. glabrata* shell; (e) mucin from bovine submaxillary glands; (f) chondroitin sulfate A from bovine trachea.

dase complex (Vector) was incubated for 30 min at room temperature. After rinsing with PBS, the staining was carried out with 0.08% (w/v) DAB, 0.075% (w/v) $NiCl_2$, and 0.01% (v/v) H_2O_2 in Tris-buffered solution, pH 7.4, for 20 min at room temperature. Controls: (1) without lectin, (2) without ABC, (3) without DAB, (4) lectins preincubated with their specific sugar.

Results

Hexosamine quantification and infrared spectrometry

In this preparation, the IM of *B. glabrata* included the periostracum. Of the hydrolyzable part of the IM (63.5% [w/w]), 3.4% (w/w) was composed of hexosamines. After a previous alkaline treatment, alkali-resistant hexosamines represented 2.9% (w/w) of the IM—that is, 85% (w/w) of the total hexosamines. The hexosamines of the SM were not alkali-resistant.

The finding of alkali-resistant hexosamines in the IM hinted at the occurrence of chitin. The infrared absorption spectra of the IM and SM of the *Biomphalaria glabrata* shell (Fig. 1) were examined to see whether chitin was visible in the IM and whether the occurrence of GAGs in the SM could be confirmed. The main absorption bands are listed in Table I.

The pattern of the IM (Fig. 1a) differed from that of

Table I

Positions of the main infrared absorption bands of the extracted water-soluble organic matrix fraction of the Biomphalaria glabrata *shell (SM), mucin from bovine submaxillary glands, chondroitin sulfate A from bovine trachea, the water-insoluble matrix fraction (IM), the alkali-treated protein-free IM, and chitin from crabshell; appropriate functional groups are suggested in the left column*

Functional group	SM	Mucin	Chondroitin sulfate	IM	Protein-free IM	Chitin
-OH/H_2O	3336	3395	3436	3309	(3463)	3451
-COOH	2933	2927	2927	2930	—	2884
Amide I	1657	1655	1616*	1656	1659	1656
Amide II	1534	1546	—	1528	1553	1562
-COO^- symm.	1448	1441	—	—	—	—
	1404	—	1412	1410	—	—
C-H bending	—	1377	1376	—	—	1376
Amide III	1311	1316	—	—	—	1307
Sulfate	1241	1238	1241	1228	—	—
-C-O single bonding	—	—	—	—	1214	(1201)
	—	—	—	1153	1152	1158
-OH sugar	—	1124	—	—	—	1113
	1066	—	1065	1073	—	1067
	—	1042	—	—	—	1030
Sulfate	—	—	926	—	—	—
	—	—	855	—	—	—
Amide IV	—	—	—	622	623	—

( ) = weak band.
* Probably due to *N*-acetyl group.

the soluble matrix (Fig. 1d). The IM showed a strong protein peak (amide I) at 1656 cm^{-1} and a medium band (amide II) at 1528 cm^{-1}. Although no amide III band and just one symmetric COO^- vibration appeared, a band indicating a -C-O bonding came out at 1153 cm^{-1}. The band at 1228 cm^{-1} may point to sulfate, and the absorption at 1073 cm^{-1} to carbohydrates. Any chitin present should have become apparent in the IM samples from which protein had been removed (Fig. 1b), but this was not the case. A comparison with chitin from crabshell (Fig. 1c) showed a strikingly different pattern. The alkaline treatment removed the carboxylic, hydroxylic, and sulfate bands preferentially, so that the "unmasked" residue of the insoluble matrix probably consisted of sclerotized proteins only.

Table II

The gas-chromatographically identified sugars in the extracted water-soluble (SM) and insoluble (IM) organic matrix fractions of the Biomphalaria glabrata *shell*

	SM	IM
Glucose	+	+
Galactose	+	+
Mannose	+	+
N-Acetyl-Glucosamine	+	+
N-Acetyl-Galactosamine	+	−
Small unidentified peaks	2 plus 1	2

The SM (Fig. 1d) showed a strong protein peak (amide I) at 1657 cm^{-1}, a medium band (amide II) at 1534 cm^{-1}, and a weak band (amide III) at 1311 cm^{-1}. The absorption bands at 1448 and 1404 cm^{-1} were probably due to carboxylic groups, while carbohydrates were responsible for the band at 1066 cm^{-1}. Since sulfate is indicated by bands between 1250 and 1230 as well as 925 and 850 cm^{-1}, the band at 1241 cm^{-1} can be interpreted as a sulfate band. In contrast, the pure GAG chondroitin sulfate A (Fig. 1f) contained no amide bands—the band at 1616 cm^{-1} was probably due to *N*-acetyl groups located on the galactosamine—but all sulfate bands. Mucin from bovine submaxillary glands (Fig. 1e), which consists of protein-bound GAGs, showed amide bands very similar to those of the soluble shell matrix: just one sulfate band at 1238 cm^{-1}, one symmetric carboxylic band, but two different carbohydrate bands. The infrared spectra from bovine mucin and the extracted shell matrix were almost identical, indicating the occurrence of protein-bound GAGs in the shell.

Carbohydrates of the organic matrix

The sugars of the soluble and the insoluble shell matrix of *B. glabrata* were identified gas-chromatographically as listed in Table II. In the SM and IM, two identical plus one additional peak in the SM remained unidentified. These unidentified peaks were not *N*-acetyl-mannosamine, arabinose, fucose, fructose, galactosamine, galacturonic acid,

Table III

The binding pattern of lectins to the extracted water-soluble (SM) and insoluble (IM) organic matrix fractions of the Biomphalaria glabrata *shell*

Lectin	Specificity*	Inhibitor	SM	IM
UEA-I	L-Fuc $\alpha 1 \rightarrow 2$ Gal	—	−	n.t.
LTA	L-Fuc $\alpha 1 \rightarrow 2$ Gal	—	−	n.t.
DBA	GalNac $\alpha 1 \rightarrow 3$ GalNac	—	−	−
SBA	GalNac α or $\beta 1 \rightarrow 3$ Gal	1.0 *M* GalNac	(+)	−
MPA	Gal $\beta 1 \rightarrow 3$ GalNac > GalNac $\alpha 1 \rightarrow 6$ Gal	0.5 *M* Gal or 0.5 *M* GalNac	+++	+++
PNA	Gal $\rightarrow \beta 1 \rightarrow 3$ GalNac	0.025 *M* Gal	(+)	−
RCA1	Gal $\beta 1 \rightarrow 4$ GlcNac	0.050 *M* Gal	+	(+)
ConA	Man $\alpha 1 \rightarrow$ > Glc $\alpha 1 \rightarrow$	0.100 *M* Man	++	+++
WGA	$(GlcNac\ \beta 1 \rightarrow 4)_n$ $(n = 5 > 4 > 3 > 2)$	0.005 *M* Chitotriose	++	+++

Lectins: UEA-I = *Ulex europaeus* agglutinin; LTA = *Lotus tetragonolobus* agglutinin; DBA = *Dolichus biflorus* agglutinin; SBA = *Glycine max* agglutinin; MPA = *Maclura pomifera* agglutinin; PNA = *Arachis hypogaea* agglutinin; RCA1 = *Ricinus communis* agglutinin; ConA = *Concanavalia ensiformis* agglutinin; WGA = *Triticum vulgaris* agglutinin.

* Preferred specificity according to Wu *et al.* (1988).

n.t. = not tested.

(+) = weak bond.

glucosamine, glucuronic acid, mannosamine, mannuronic acid, rhamnose, or xylose. The recovery of uronic acids was generally poor.

Terminal sugar moieties were identified with the help of lectins, binding to the water-soluble and water-insoluble matrix (Table III). No terminal fucose was observed. The GalNac-specific lectins DBA and SBA were negative or just weakly positive. The high amount of sugar, which was necessary to inhibit the binding of SBA, indicated an unspecific binding. Since MPA with a lower preference also binds to GalNac, the occurrence of this sugar in a terminal position cannot be absolutely excluded for the SM. The strong binding of MPA, in combination with the weakly positive reactions of PNA and RCA1, indicated that both the SM and the IM contained terminal galactosyl moieties. The binding of ConA can be due to glucose or mannose, which were both found to be constituents of the matrix (Table II). Also, terminal GlcNac was identified in both matrix fractions by the binding of WGA.

The binding of lectins to glycoproteins and proteoglycans of the organic matrix after isoelectrical focusing is demonstrated in Figure 2. The matrix protein of *B. glabrata* with a size of 19.6 kDa and a pI of 7.4 was bound by ConA only, referring to terminal glucosyl or mannosyl moieties. ConA with a lower preference also binds to GlcNac, but the occurrence of this sugar in terminal position at the 19.6 kDa protein is excluded because of the negative reaction with WGA. Furthermore, the 19.6 kDa protein was not bound by PNA and RCA1 (Table IV)—results that were obtained after SDS electrophoresis and blot transfer (not shown).

In the acidic range between pI 5.4 and 3.3, where the alcian-blue-positive material was found, ConA, bound to two bands at pI 5.0 and 4.5, indicated terminal glucosyl or mannosyl moieties. WGA, bound to a band with a pI of 3.5, indicated terminal GlcNac; MPA, bound to 10 bands in the range between pI 4.5 and 3.3, indicated terminal galactosyl or GalNac moieties.

Carbohydrates at the mantle edge

The appearance of mucus at the shell-forming mantle edge was detected by alcian blue staining. All three fixations gave satisfactory results, but the use of CPC (method 2) resulted in the best preservation of lighter stained minor amounts of mucus, especially in the belt and the outer mantle epithelium (OME). The results are combined in a schematic drawing (Fig. 3). Staining at pH 2.5, which indicates carboxylic groups, gave a strong reaction with mucus cells at the base of the PG, a weaker reaction in the middle of the belt, and was seen as a thin apical layer at the cells of the OME. Furthermore, mucus cells, which secrete their contents towards the inner mantle epithelium (IME), gave strong positive reactions with alcian blue at pH 2.5 and 1.0, indicating carboxylated as well as sulfated mucus.

The results of all lectins applied to the shell-forming mantle edge and the shell matrix of *B. glabrata* are summarized in Table IV. The lectins UEA-I, SBA, RCA1, PNA, ConA, and WGA bound specifically to the periostracum groove (PG) (Zone 1). All lectins reacted, with different strengths, with the distal belt (Zones 2 and 3). The binding of SBA (Fig. 4), WGA (Fig. 5), and ConA (Fig. 6) is shown. At the proximal belt (Zone 4) and at the OME (Zone 5), a thin layer of material that reacted with WGA (Fig. 5) and ConA (Fig. 6) was seen, but only apically. The mucus cells of the IME reacted almost like

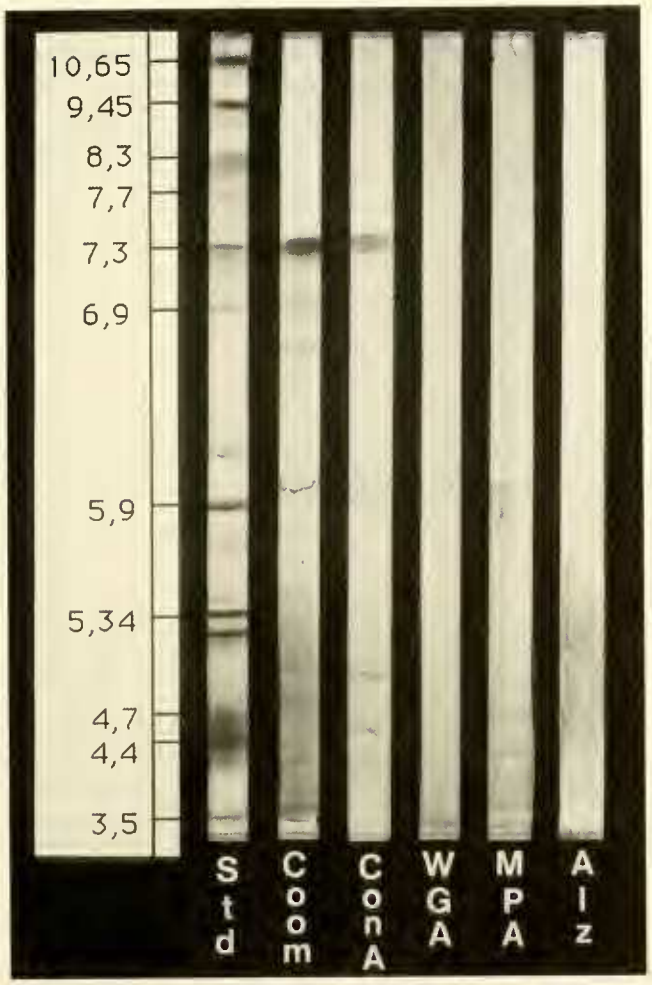

Figure 2. Isoelectrical focusing of the extracted water-soluble organic matrix fraction of the *Biomphalaria glabrata* shell and application of lectins. Std = standard proteins; Coom = matrix, stained with Coomassie brilliant blue; ConA, WGA, and MPA = lectins; Alz = matrix, stained with alcian blue.

the belt. The calcium cells in the interstitium bound almost all lectins, but no inhibition by the specific sugars occurred, indicating merely an unspecific reaction.

Discussion

Chitin

The hexosamines of the IM were mainly alkali-resistant, so the matrix could contain chitin. The infrared spectrometry could not, however, confirm this assumption (Fig. 1b). Bielefeld *et al.* (1993a), using electron microscopy, found WGA binding sites in the periostracum of *B. glabrata*. After a chitinase digestion, the reactivity of these sites was reduced, but not negative. The cells at the PG and the belt, however, were not affected by chitinase. The results indicate that chitin is one of the GlcNac-positive components of the periostracum of *B. glabrata*, but the amount may be considered rather low.

Glycoproteins and proteoglycans

Prominent among the proteins of the SM of *B. glabrata* is one that has a size of 19.6 kDa and an isoelectrical point of 7.4. *N*-terminal microsequencing revealed that 15 or 16 of the 24 amino acids identified in the 19.6-kDa protein were hydrophobic (Marxen and Becker, 1997). Because of its high pI, this protein cannot be directly involved in the binding of calcium. As demonstrated by the binding of lectins to the IEF gel (Fig. 2, Table IV), this protein is glycosylated with glucosyl and mannosyl moieties, singly or in combination. Thus, this glycoprotein contains hydrophobic as well as hydrophilic domains and may have evolved from a membrane protein. In the SM of *Mytilus edulis*, Keith *et al.* (1993) found a protein with a size of 21 kDa and a highly hydrophobic *N*-terminus with a sequence that was identical in the positions 7, 8, and 9 to that of the 19.6-kDa protein of *B. glabrata*. It is not known, however, whether the 21-kDa protein from *M. edulis* is glycosylated. Mann *et al.* (1988) observed a change in the modification of calcium carbonate crystals under a stearic acid monolayer. The hydrophobic and hydrophilic parts of the 19.6-kDa protein (and perhaps also the 21-kDa protein from *M. edulis*) could give molecules of this kind a detergent-like quality, by which they might—among other possible functions—play a role in the determination of the crystal modification.

The acidic material of the SM of *B. glabrata* shows a variety of possible terminal sugar moieties at various isoelectrical points (Fig. 2, Table IV). Although the main part with a broad range of isoelectrical points is bound by the Gal- or GalNac-specific MPA, only a very acidic component is detected by the GlcNac-specific WGA. A lower acidic part is detected by ConA, pointing to Man or Glc, which are not common sugars in GAGs. The results indicate the occurrence of several different GAGs and glycoproteins. Mixtures of GAGs are common in vertebrates (Volpi, 1996) as well as in molluscan tissues (Dietrich *et al.*, 1983). Cottrell *et al.* (1994) detected a large number of hexoses and hexosamines in the body mucus of the slug (*Arion ater*) and showed that—in addition to the main fraction, which probably is heparan sulfate—other, unidentified GAGs unknown in vertebrates must also be present. Moreover, in invertebrates the variable glycosylation of one core protein is possible (Har-El and Tanzer, 1993).

Mucopolysaccharides have been found in other molluscan shells as well (Simkiss, 1965; Worms and Weiner, 1986), but their function in the shell remains questionable. Sulfated polysaccharides have been discussed as possible calcium-binding sites (Wilbur, 1976) and, because of their appearance in the center of nacreous tablets, could play a role in the nucleation and the growth inhibition of the mineral (Crenshaw and Ristedt, 1976).

Table IV

The binding pattern of lectins to the periostracum groove (PG), the belt, and the outer mantle epithelium (OME) of Biomphalaria glabrata, *the extracted water-soluble organic matrix fraction (SM), the 19.6-kDa protein, and the acid IEF bands of the* B. glabrata *shell*

Lectin	Specificity	PG	Belt	OME	Whole SM	19.6 kDa protein	Acidic IEF bands
UEA-I	Fuc	(+)	(+)	−	−	n.t.	n.t.
DBA	GalNac	−	(+)	−	−	n.t.	n.t.
SBA	GalNac	++	+++	−	(+)	n.t.	−
MPA	Gal, GalNac	−	++	−	+++	−	+++
PNA	Gal	+	+	−	(+)	−	n.t.
RCA1	Gal	(+)	++	−	+	−	n.t.
ConA	Man, Glc	++	+++	$+_a$	++	++	+
WGA	GlcNac	+++	++	$+_a$	++	−	++

Lectins = UEA-I: *Ulex europaeus* agglutinin; DBA = *Dolichus biflorus* agglutinin; SBA = *Glycine max* agglutinin; MPA = *Maclura pomifera* agglutinin; PNA = *Arachis hypogaea* agglutinin; RCA1 = *Ricinus communis* agglutinin; ConA = *Concanavalia ensiformis* agglutinin; WGA = *Triticum vulgaris* agglutinin.

n.t. = not tested.

a = positive reaction apically.

(+) = weak bond.

Histological observations

In the shell-forming tissue, alcian-blue-positive material was found in cells of the PG, the belt and, as a thin apical border, the OME (Fig. 3). Surprisingly, no sulfated mucopolysaccharides were detected here, although a considerable amount of sulfate is found in the shell matrix (Crenshaw, 1972; Marxen and Becker, 1997). Also, in the freshwater mussel *Anodonta californiensis*, sulfate was observed in those parts of the mantle edge that correspond to the IME of *B. glabrata*, but not in the outer fold, which corresponds to the belt and OME (Hovingh and Linker, 1993). Thus, the origin of the sulfate in the organic shell matrix remains questionable.

Eight lectins reacted positively—most strongly so—with the belt (Zones 2 and 3), although their reactions with the PG were generally weaker or partly negative, indicating that the sugar concentrations were higher in the belt. The question remains, why GalNac-specific lectins, especially SBA, bound to the shell-forming tissue but not to the organic matrix of the shell.

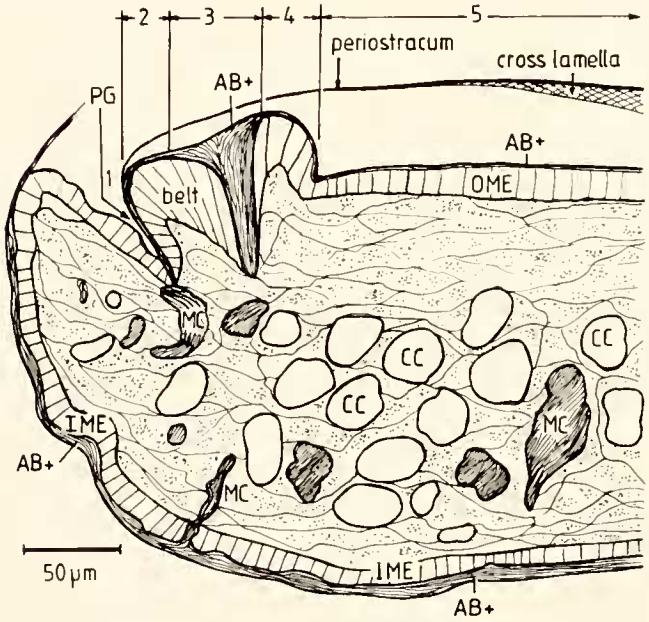

Figure 3. Drawing of the shell-forming mantle edge of *Biomphalaria glabrata*, showing the combined results after fixing the tissue with three different methods and staining with alcian blue. Zones 1–5 are facing the shell. A strongly positive reaction product appears in the mucus cells (MC) and the periostracum groove (PG) after staining with alcian blue at pH 2.5 (AB+, hatched areas). A more lightly stained reaction product descends to the basis of the high prismatic cells of the belt and is detected apically in the cells of the outer mantle epithelium (OME). A thick layer of mucus secreted from the mucus cells can be seen on the cells of the inner mantle epithelium (IME). The tissue is filled with calcium cells (CC).

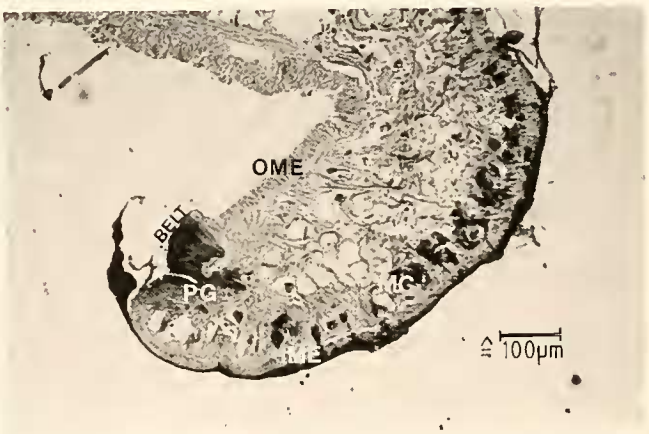

Figure 4. Binding of the lectin SBA (1:200) to the mantle edge of *Biomphalaria glabrata*. A positive reaction product is visible in the cells of the periostracum groove (PG), Zones 2 and 3 of the belt, mucus cells (MC) near the inner mantle epithelium (IME), and at a mucous border on the IME. No reaction product is found in the outer mantle epithelium (OME).

All the glycosylated components of the matrix can be produced in cells of the PG (Zone 1) and the distal belt (Zones 2 and 3). In contrast to Zones 1 to 3, the proximal belt (Zone 4) and the OME (Zone 5) exhibited only terminal GlcNac and Man/Glc, respectively pointing to the production of a GAG with a pI of 3.5 and to the less acidic glycosylated material, the 19.6-kDa glycoprotein, or both. The lectin binding pattern of the mantle edge indicates a functional differentiation among the various kinds of GAGs, but the same GAG has different functions depending on the location of its production.

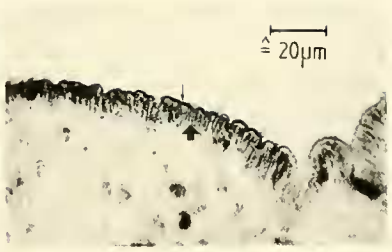

Figure 6. Binding of the lectin ConA (1:200) to the outer mantle epithelium of *Biomphalaria glabrata*. The positive reaction product can be seen apically (small arrow); the gray appearance of the cell bodies is caused by their natural orange color (large arrow).

Conclusions

The striking difference in the lectin binding pattern between the distal part (Zones 1 to 3) and the proximal part (Zones 4 and 5) of the shell-forming tissue gives new emphasis to a strict functional separation between these parts.

In Zones 1, 2, and 3 of the mantle edge of freshwater snails, a phenol oxidase activity has been observed (Timmermanns, 1969; Bielefeld *et al.*, 1993a). This enzyme may be responsible for the sclerotization and tanning of the periostracum (Waite, 1984) and the matrix (Gordon and Carriker, 1980), which thus become water-insoluble. The sugar patterns in the SM and IM of *B. glabrata* are very similar, indicating that GAGs are trapped in the network of sclerotized proteins.

In Zones 4 and 5, a strong alkaline phosphatase activity (Timmermanns, 1969; Bielefeld *et al.*, 1993b) and a carbonic anhydrase activity were detected (Timmermanns, 1969; Boer and Witteveen, 1980); both enzymes are thought to be closely related to the mineralization process (Wilbur, 1964; Watabe, 1984). Because calcium has also been localized at these proximal zones in *B. glabrata* (Bielefeld *et al.*, 1992), this site can be considered to be the region where the calcification of the organic matrix of the shell takes place.

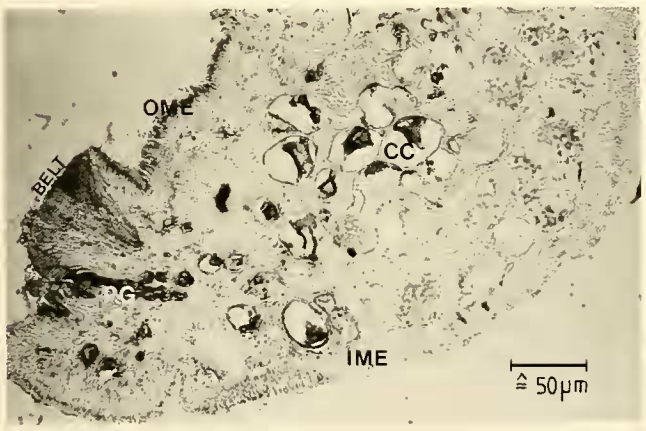

Figure 5. Binding of the lectin WGA (1:200) to the mantle edge of *Biomphalaria glabrata*. A positive reaction product shows in the cells of the periostracum groove (PG), Zones 2 and 3 of the belt, apically in the cells of the transitional Zone 4 and the outer mantle epithelium (OME). The mucus cells near the inner mantle epithelium (IME) are slightly stained, and the calcium cells (CC) of the interstitium give an unspecific reaction.

The periostracum is produced by the groove and the distal belt (Bielefeld *et al.*, 1993a). Furthermore, the belt seems to be the production site for those GAGs and proteins that form the structural framework of the organic matrix. Here, the proteins will be partly linked by the phenol oxidase, trapping acidic polysaccharides. Matrix constituents that may be directly involved in the calcification process of the shell seem to be produced, in addition, by the mineralizing region of the mantle. In *B. glabrata*, these constituents presumably include the very acidic WGA-positive part of the GAGs, the less acidic ConA-positive material, and the 19.6-kDa protein. Because components of the SM are known to enhance crystal nucleation when immobilized but inhibit crystal growth when free in solution (*e.g.*, Wheeler and Sikes, 1989), the function of the acidic polysaccharides may vary depending on their place of origin. Distally produced, immobilized GAGs may provide nucleation sites, while the proximally produced ones could instead be involved in regulating crystal growth.

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